

Identification, characterization and genetic mapping of TLR7, TLR8a1 and TLR8a2 genes in rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

Induction of the innate immune pathways is critical for early anti-viral defense but there is limited understanding of how teleost fish recognize viral molecules and activate these pathways. In mammals, Toll-like receptors (TLR) 7 and 8 bind single-stranded RNA of viral origin and are activated by synthetic anti-viral imidazoquinoline compounds. Herein, we identify and describe the rainbow trout (*Oncorhynchus mykiss*) TLR7 and TLR8 gene orthologs and their mRNA expression. Two TLR7/8 loci were identified from a rainbow trout bacterial artificial chromosome (BAC) library using DNA fingerprinting and genetic linkage analyses. Direct sequencing of two representative BACs revealed intact *omTLR7* and *omTLR8a1* open reading frames (ORFs) located on chromosome 3 and a second locus on chromosome 22 that contains an *omTLR8a2* ORF and a putative TLR7 pseudogene. We used the *omTLR8a1/2* nomenclature for the two trout TLR8 genes as phylogenetic analysis revealed that they and all the other teleost TLR8 genes sequenced to date are similar to the zebrafish TLR8a, but are distinct from the zebrafish TLR8b. The duplicated trout loci exhibit conserved synteny with other fish genomes extending beyond the tandem of TLR7/8 genes. The trout TLR7 and 8a1/2 genes are composed of a single large exon similar to all other described TLR7/8 genes. The *omTLR7* ORF is predicted to encode a 1049 amino acid (aa) protein with 84% similarity to the *Fugu* TLR7 and a conserved pattern of predicted leucine-rich repeats (LRR). The *omTLR8a1* and *omTLR8a2* are predicted to encode 1035- and 1034-aa proteins, respectively, and have 86% similarity to each other. *omTLR8a1* is likely the ortholog of the only Atlantic salmon TLR8 gene described to date as they have 95% aa sequence similarity. The tissue expression profiles of *omTLR7*, *omTLR8a1* and *omTLR8a2* in healthy trout were highest in spleen tissue followed by anterior and then posterior kidney tissues. Rainbow trout anterior kidney leukocytes produced elevated levels of pro-inflammatory and type I interferon cytokines mRNA in response to stimulation with the human TLR7/8 agonist R848 or the TLR3 agonist poly I:C. Only poly I:C-induced *IFN2* transcription was significantly suppressed in the presence of chloroquine, a compound known to block endosomal acidification and inhibit endosomal maturation. The effect of chloroquine on R848-induced cytokine expression was equivocal and so it remains questionable whether rainbow trout recognition of R848 requires endosomal maturation. TLR7 and TLR8a1 expression levels in rainbow trout anterior kidney leukocytes were not affected by poly I:C or R848 treatments, but surprisingly, TLR8a2 expression was moderately down-regulated by R848. The down-regulation of *omTLR8a2* may imply that this gene has evolved to a new or altered function in rainbow trout, as often occurs when the two duplicated genes remain active.

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1. Introduction

Receptors that recognize conserved pathogen molecules are part of the ancient innate arm of the immune system and are conserved in both invertebrate and vertebrate lineages. Toll-like receptors (TLRs) are a family of transmembrane proteins that recognize conserved pathogen structures to induce immune effector molecules. In vertebrates, TLRs can distinguish among classes of pathogens and serve an important role in orchestrating

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the appropriate adaptive immune responses [1]. TLRs are type I membrane proteins that contain an extracellular N-terminus with leucine-rich repeat region (LRR) and an intracellular C-terminus with a Toll/IL-1 receptor domain (TIR). The cytoplasmatic TIR domain harbors conserved amino acids that have been shown to be involved in the signaling as well as in the localization of the TLR [2,3], while the LRR region is involved in pathogen recognition [4]. Approximately 10 TLRs have been described in several species, and in mammals most of the TLRs have been shown to identify distinct pathogen associated components [5–8].

TLR3, 7, 8 and 9 are the members of the nucleic acid subgroup of the TLR gene family. In mammals, TLR3 has been shown to respond to double-stranded RNA (dsRNA), TLR9 to unmethylated CpG DNA and TLR7 and TLR8 were shown to be activated by synthetic anti-viral imidazoquinoline compounds and were implicated in recognizing single-stranded RNA [9–13]. These TLRs are primarily located in the endoplasmic reticulum and in lysosomal-like vesicles and are thought to have an important role in anti-viral immunity [14]. The TLR7, 8 and 9 genes form a phylogenetically related cluster based on sequence similarities and genomic structures [7,8]. Upon activation they recruit myeloid differentiation primary response protein 88 (MyD88) that through several effector molecules initiates the activation of two major signaling pathways resulting in the production of pro-inflammatory cytokines and/or type I interferons [1,15]. The teleost specific TLR22 was recently shown to recognize long-sized dsRNA, but unlike the other members of this TLR gene family subgroup it is primarily located on the cell surface [16].

TLR orthologs have been described in several fish species. A complete repertoire of 10 or more TLRs was identified in the *Fugu* and the zebrafish genomes [17–19]. A number of TLR genes were identified, characterized and mapped in rainbow trout including TLR3, TLR5, TLR5S, TLR9, TLR20 and TLR22 [20–25]. Although found in all the teleost genomes sequenced to date, rainbow trout TLR7 and TLR8 orthologs have not been reported. Due to their important role in anti-viral immunity we hypothesized that the rainbow trout genome contains TLR7 and TLR8 orthologs.

In this paper, we report the TLR7 and TLR8 orthologs in rainbow trout (*Oncorhynchus mykiss*) and describe their genomic location, gene organization, expression pattern, and regulation. Rainbow trout are widely used in basic research [26] and they are economically important for aquaculture and sport fishing. A better understanding of anti-viral immunity is necessary to reduce viral disease loss in aquaculture and for comparative study of immune system evolution in teleost fish.

2. Materials and methods

2.1. Genes identification and sequencing

The rainbow trout gene index (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=r_trout) [27] and Atlantic salmon gene index (<http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=salmon>) [28] were screened by BLAST for ESTs with high homology to *Fugu* TLR8 (Accession AC156438) [7]. Two Atlantic salmon ESTs were identified (Accessions DY707798 and DW540423). PCR primers were designed from conserved gene segments (Supplement Table 1) for screening of the NCCCWA Swanson 10× bacterial artificial chromosome (BAC) library PCR super-pools as previously described [29]. BACs identified as positive for TLR8 were fingerprinted using HindIII as previously described [30] to identify sets of overlapping clones. BAC DNA was isolated following a miniprep protocol (Qiagen, Valencia, CA). Following HindIII digestion and gel electrophoresis, gel images were captured using a Molecular Dynamics Typhoon 9210 Variable Mode Imager and exported as TIF files. Banding patterns were

analyzed using Image 3.10 and FPC (Fingerprinted Contigs) V6 software [31] to assemble overlapping BACs into contigs [32]. The positive BACs were assembled into two contigs and DNA from two representative BAC clones (141K11 and 318O13) was isolated using a Large Construct Kit (Qiagen, Valencia, CA), according to the manufacturer's protocol. The primer walking method [24,29] was used to obtain genomic sequence directly from the BACs. The samples were sequenced on an ABI 3100 Automated Sequencer (ABI, Foster City, CA). PCR primers were designed from the putative genomic open reading frames (ORFs) to amplify spleen cDNA. The PCR amplicons were gel-purified (Qiagen, Valencia, CA) and sequenced. The genomic and cDNA sequences were aligned using Sequencher (Gene Codes Corporation, Ann Arbor, MI) to detect intron/exon boundaries. A 5' RACE protocol was performed in spleen RNA samples (1 µg/µL) using the GeneRacer kit (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Two nested primers were designed for each gene. PCR reactions were set-up as follow: 1 µL template cDNA (25 ng/µL), 2 µL buffer (20 mM Mg), 1 µL MgCl (10 mM), 2 µL dNTP (200 mM), 2 µL each primers (10 µM), 0.4 µL Pfu Taq (Invitrogen, Carlsbad, CA). The PCR products were isolated from the agarose gel using the QIAquick kit (Qiagen, Valencia, CA) and sequenced.

2.2. Microsatellite markers isolation, genotyping and genetic linkage analysis

A shotgun library was prepared from each of the two representative BAC clones from each contig and 96–192 sub-clones were sequenced to isolate microsatellite genetic markers as previously described [33]. The sub clone sequences were used in BLAST sequence similarity searches for identifying neighboring genes and conducting comparative genomics analyses. Putative TLR7 sequences from each BAC were used for primer walking to obtain putative TLR7 ORFs.

The NCCCWA mapping panel of five families was genotyped with microsatellites as previously described [34]. Two microsatellites (GenBank Accessions GF100699 and GF100700) were genotyped using the tailed protocol of Boutin-Ganache et al. [35]. Primers were obtained from commercial sources (Alpha-DNA, Montreal, Quebec, Canada). Three oligonucleotide primers were used in each DNA amplification reaction (forward: 5' GAGTTTCC-CAGTCACGAC-primer sequence 3'; reverse: 5' GTTT-primer sequence 3'; fluorescent labeled primer with FAM: 5' GAGTTTCC-CAGTCACGAC 3'). Primers were optimized for amplification by varying annealing temperatures and MgCl₂ concentrations. PCR reactions (12 µL total volume) included 50 ng DNA, 1.5–2.5 mM MgCl₂, 2 pmol of forward primer, 6 pmol of reverse primer, 1 pmol of fluorescent labeled primer, 200 µM dNTPs, 1× manufacturer's reaction buffer, and 0.5 unit Taq Polymerase (ABI, Foster City, CA). Amplifications were conducted in an MJ Research DNA Engine thermal cycler model PTC 200 (MJ Research, Waltham, MA) as follows: an initial denaturation at 95 °C for 10 min, 30 cycles consisting of 94 °C for 60 s, annealing temperature for 45 s, 72 °C extension for 45 s; followed by a final extension of 72 °C for 10 min. PCR products were visualized on agarose gels after staining with ethidium bromide. Three microliters of each PCR product was added to 20 µL of water, 1 µL of the diluted sample was added to 12.5 µL of loading mixture made up with 12 µL of HiDi formamide and 0.5 µL of Genscan 400 ROX internal size standard. Samples were denatured at 95 °C for 5 min and kept on ice until loading on an ABI 3730 DNA Analyzer (ABI, Foster City, CA). Output files were analyzed using GeneMapper version 3.7 (ABI, Foster City, CA), formatted using Microsoft Excel and stored in a Microsoft Access database.

The two microsatellites were placed on the rainbow trout genetic map by two-point linkage analysis as previously described

[20,22,34]. Genotype data combined for both sexes were formatted using MAKEPED of the LINKAGE [36] program and checked for inconsistencies with Mendelian inheritance using PEDCHECK [37]. RECODE [38] and LNKTOCRI [39] were used to assemble the data into CRIMAP [40] format. Genotype data were added to the current NCCWA genetic map [34] and MULTIMAP [41] was used to conduct two-point linkage analyses to identify the closest markers from the published map having the highest LOD scores.

2.3. Peptides sequence alignment and comparative genomic syntenic analyses

The ORFs and their translated amino acids sequences were predicted using the ExPASy Translate tool (<http://us.expasy.org/>). Homologous genes from other species were identified for *omTLR7* and *omTLR8* using BLASTX global alignment (Table 1). Amino acid sequences were aligned using ClustalW [42] (<http://www.ebi.ac.uk/clustalw/>) and this alignment was used for phylogenetic analysis in the program MEGA3 [43] (<http://www.megasoftware.net/>). A consensus phylogenetic tree was generated using the neighbor-joining algorithm (Poisson correction/exclusion of gaps) and support for the tree was determined using 10,000 repetitions of bootstrap analysis. Syntenic relationships were deduced by identifying the genome locations of putative homologs in the genome sequences of zebrafish, stickleback, medaka and Tetraodon using the Ensembl Genome Browser (<http://www.ensembl.org/>). The simple modular architecture research tool (SMART) ([44]; <http://smart.embl-heidelberg.de/>) and SignalP 3.0 ([45]; <http://www.cbs.dtu.dk/services/SignalP/>) were used to characterize the peptides architecture based on the amino acid sequence.

2.4. Tissue samples and cell cultures

A range of tissue samples were obtained from five naive 10-month-old female rainbow trout, with an average weight of 397.4 ± 16.4 g and average length of 298.4 ± 2.5 cm. Fish were euthanized with an overdose of tricaine methanesulfonate (>250 mg/L, Western Chemical, Ferndale, WA). The tissues collected included: brain, pituitary, heart, liver, anterior kidney, posterior kidney, spleen, stomach, intestine, pyloric ceca, skin, fin tissue, gill filaments, brachial arch cartilage, eye, white muscle, red muscle, and abdominal adipose tissues (fat). All tissues were flash frozen in liquid nitrogen and stored at -80°C until RNA isolation.

Anterior kidney leukocytes for cell culture stimulations were obtained from four healthy adult rainbow trout (800–900 g). Fish were euthanized by submerging them in 200 mg MS-222 (Sigma–

Aldrich Corp., St. Louis, MO)/liter water bath, and anterior kidneys were harvested. The kidneys were ground using mechanical tissue grinders and cell suspensions were passed through sterile mesh pore size $70\ \mu\text{m}$. Leukocyte were then isolated from the cell suspension by Histopaque-1077 (Sigma–Aldrich Corp., St. Louis, MO) density centrifugation. Cells were washed and counted using hemocytometer and resuspended into a final concentration of 1×10^7 cells/mL. One ml of the leukocyte preparation was cultured with medium alone, chloroquine (Sigma–Aldrich Corp., St. Louis, MO) ($0.5\ \mu\text{M}$; following [46]), poly I:C (Sigma–Aldrich Corp., St. Louis, MO) ($5\ \mu\text{g/mL}$), R848 (InvivoGen, San Diego, CA) ($10\ \mu\text{g/mL}$), chloroquine + poly I:C or chloroquine + R848. Cells were first added with or without chloroquine and incubated for 30 min prior to adding any other reagents to the appropriate cell samples. Cells were incubated for 6 h and washed with medium, centrifuged and the medium was removed prior to freezing at -80°C . This experiment was conducted in a similar fashion two independent times (experiments 1 and 2).

2.5. RNA isolation and reverse transcription

Total RNA was isolated from the rainbow trout tissues and the *in vitro* anterior kidney leukocyte preparations from experiment 1 using TRI-Reagent (Sigma–Aldrich Corp., St. Louis, MO) modification of the guanidine isothiocyanate/phenol–chloroform method [47]. The RNA was dissolved in $20\ \mu\text{L}$ of nuclease free water and subjected to DNase treatment following the manufacturer's protocol (DNase RQ-1, Promega, WI) to remove any genomic DNA contamination. The DNase treatment was followed with a re-extraction with TRI-Reagent to remove all residual DNase activity. The quantity of RNA was estimated using a GeneQuant RNA/DNA Calculator (BioChrom Ltd., Cambridge, UK). The quality of the RNA was assessed by agarose gel electrophoresis with the visualization of the 28S and 18S rRNA bands. Tissues RNA samples isolated from the five rainbow trout were pooled into one sample per tissue prior to synthesis of complementary DNA (cDNA). The anterior kidney leukocyte RNA from the *in vitro* stimulation experiment 2 was obtained using the RNeasy Minikit (Qiagen, Valencia, CA). RNA was quantified using the NanoDrop 1000 (ThermoScientific, Wilmington, DE). All RNA samples were stored at -80°C .

Synthesis of cDNA was initiated with $1\ \mu\text{g}$ of total RNA was mixed with $1\ \mu\text{g}$ of random hexamer primers (Promega, Madison, WI) and heated at 70°C for 5 min. This mixture was cooled on ice before addition of the reverse transcription cocktail containing $1 \times$ RT buffer, $0.5\ \text{mM}$ of each dNTP, $25\ \text{U}$ of rRNase Inhibitor (Promega, Madison, WI) and $200\ \text{U}$ of MMLV-RT (Promega,

Table 1

Amino acid sequence identities and similarities between the rainbow trout *TLR7*, *TLR8a1* and *TLR8a2* putative peptides and *TLR7* and *TLR8* from other vertebrates and the rainbow trout *TLR3*.

TLR	Trout <i>TLR7</i>		Trout <i>TLR8a2</i>		Trout <i>TLR8a1</i>		Accession no.
	Identity (%)	Similarity (%)	Identity (%)	Similarity (%)	Identity (%)	Similarity (%)	
Trout <i>TLR7</i>	100	100	43	61	41	59	GQ422119
Trout <i>TLR8a2</i>	43	61	100	100	78	86	GQ422120
Trout <i>TLR8a1</i>	41	59	78	86	100	100	GQ422121
Fugu <i>TLR7</i>	73	84	42	60	41	59	AAW69375
Danio <i>TLR7</i>	68	81	43	62	41	60	XP_701101
Porcine <i>TLR7</i>	56	73	41	61	42	60	ABG47422
Human <i>TLR7</i>	56	73	42	60	42	61	NP_057646
Chicken <i>TLR7</i>	54	72	42	59	40	59	CAG15146
Salmo <i>TLR8</i>	42	60	79	87	93	95	NP_001155165
Fugu <i>TLR8</i>	40	58	56	73	58	73	AAW69376
Danio <i>TLR8a</i>	39	57	54	71	53	71	XP_001920594
Danio <i>TLR8b</i>	40	58	45	63	45	62	XP_001340186
Bovine <i>TLR8</i>	43	61	45	63	44	62	ABN71677
Human <i>TLR8</i>	43	61	44	63	44	63	NP_619542
Trout <i>TLR3</i>	25	42	25	41	25	41	ABE69177

Madison, WI) and nuclease free water to a total reaction volume of 40 μ L. First strand cDNA synthesis was carried out at 37 °C for 60 min followed by heating the sample to 95 °C for 5 min. Synthesis of cDNA from the anterior kidney leukocyte experiment 2 was conducted as previously described [48].

2.6. Quantitative PCR analyses of TLR genes

All real-time PCR assays were conducted using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). *TLR7*, *TLR8a1* and *TLR8a2* primers were designed using VectorNTI (Invitrogen Inc., Carlsbad, CA) and purchased from Alpha-DNA (Montreal, Canada). The *TLR3* primers were the same primers used by Rodriguez et al. [24]. To assay the TLR genes, 1 μ L of cDNA (1:5 dilution of reverse transcription reaction) was combined with 5 μ L of 2 \times SYBR[®] Green PCR master mix (Applied Biosystems, Foster City, CA). For each reaction, 6 μ L of this mixture was added to 9 μ L of the primer mix for a 15 μ L reaction containing 1 \times PCR master mix and 400 nM of each primer. The reactions were carried out with the standard reaction conditions as follows, 50 °C for 2 min, 95 °C for 10 min, then 40 cycles consisting of 95 °C for 15 s and 60 °C for 1 min. The cycling reaction was followed by a dissociation curve to verify amplification of a single product. Correct product amplification was verified by fragment size and by DNA sequencing. The development and validation of the cytokine gene assays using the TaqMan[®] technology (Applied Biosystems, Foster City, CA) were previously described [48,49]. Each sample was assayed in duplicate on a single plate. The cytokine expression assays were repeated in two independent experiments at the NCCCWA lab in Leetown, WV and the WFRC lab in Seattle, WA using aliquots from the same samples. Primer and probe sequences for the TLR and cytokine gene assays are provided in Supplement Tables 2 and 3, respectively.

Calculation of relative gene expression was performed as previously described [48] using the standard curve method. To assess PCR efficiency, serial dilutions of a standard cDNA preparation or plasmid DNA encoding the gene of interest were used to generate the standard curve for each assay plate. This curve was then used to calculate the relative abundance of each transcript in each sample (see ABI User bulletin #2). Transcript abundance values were then normalized to those of Elongation Factor 1- α (EF1 α) (TLR genes) or ARP (cytokine genes) to control differences in RNA and cDNA loading. Fold change was estimated as previously described [48]. Briefly, fold increases were calculated relative to a calibrator group (white muscle for the tissue panel or media control for *in vitro* stimulated anterior kidney leukocytes). Fold increase was calculated as $(T_s/T_n)/(C_s/C_n)$ where T_s equals the treated sample assayed for the specific gene and T_n equals the treated sample assayed for the normalizer gene and C_s and C_n equal the calibrator group with the specific and normalizing gene, respectively. The standard deviation of the mean fold increase for each group was calculated as per ABI User Bulletin #2. An ANOVA was used to evaluate differences among groups using the normalized expression values followed by a Dunn's post hoc test to compare differences among groups. All statistical analyses were done using GraphPad InStat V.3.01 statistics package (GraphPad Software, La Jolla, CA). *P*-values less than 0.05 were considered significant.

3. Results

3.1. The duplicated rainbow trout TLR7/TLR8 genomic region displays conserved synteny with other vertebrates

Screening of the rainbow trout BAC library identified a total of seven positive BACs that formed two contigs (five and two BACs) by

DNA fingerprint analysis. DNA sequencing and genetic linkage mapping were conducted on BAC clones 141K11 and 318O13 which represented the five and two BACs contigs, respectively. A 5106 bp *TLR8*-like fragment was sequenced by primer walking from BAC 141K11, revealing a single-exon putative ORF of 1034 amino acid (Genbank Accession no. GQ422120), and a 3658 bp fragment was sequenced from BAC 318O13, revealing a putative ORF of 1035 amino acids (Genbank Accession no. GQ422121). We designated the genes from BACs 318O13 and 141K11, *omTLR8a1* and *omTLR8a2*, respectively. No introns or additional 5' untranslated region (UTR) exons were detected in either of the two *TLR8* genes using PCR amplification of spleen cDNA or 5' RACE of spleen RNA. Partial sequencing of shotgun libraries that were prepared from the two BACs revealed sub-clones with sequence similarity to *TLR7*. A 4781 bp *TLR7*-like fragment was sequenced by primer walking from BAC 318O13, revealing a single-exon encoding a putative ORF of 1049 amino acids (Genbank Accession no. GQ422119). An additional 165 bp 5' UTR exon with a single transcription start site was detected using 5' RACE (data not shown). The ORF of the *TLR7*-like sequences from BAC 141K11 was disrupted by transposon sequences (Accessions F1978339 and F1978361) indicating that the *TLR7*-like sequence is a pseudogene.

Local BLASTP alignments showed highest amino acid sequence similarity of the putative *omTLR7* to the *Fugu TLR7* (84%) while *omTLR8a1* had greater similarity with Atlantic salmon *TLR8* (95%) than with *omTLR8a2* (86%) (Table 1). Phylogenetic analysis further supported our proposed annotations: *omTLR7* clustered within the same clade of other vertebrate *TLR7* peptides and *omTLR8a1* and *omTLR8a2* clustered with other teleosts *TLR8* putative peptides (Fig. 1). Interestingly, while the zebrafish *TLR8a* clustered with the other teleosts *TLR8* peptides the zebrafish *TLR8b* was as separated from the teleosts *TLR8* clade as it was from the mammalian *TLR8* clade. The high similarity of *omTLR8a1* to the salmon *TLR8* [50] suggests that they are orthologs and that an ortholog of *omTLR8a2* has not yet been described from Atlantic salmon.

Two microsatellite markers were isolated and assigned to chromosomes on the rainbow trout genetic linkage map [34]. Marker *OMM3183* (Accession GF100699) was isolated from BAC 141K11 near *omTLR8a2* and was mapped to chromosome 22. It was

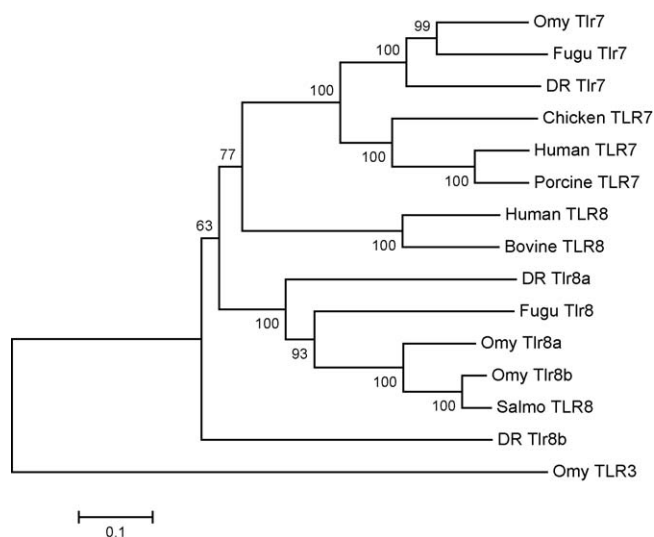


Fig. 1. Phylogenetic tree of *Tlr7* and *Tlr8* with rainbow trout *Tlr3* as an out-group. Accession numbers of the sequences are given in Table 1. Amino acid sequences were aligned using ClustalW and phylogenetic trees were then constructed using the neighbor-joining method. Bootstrap values indicate the percentage of times that a given node could be recovered in 10,000 replications and are indicated at the branch points. Omy: rainbow trout; DR: zebrafish; Salmo: Atlantic salmon.

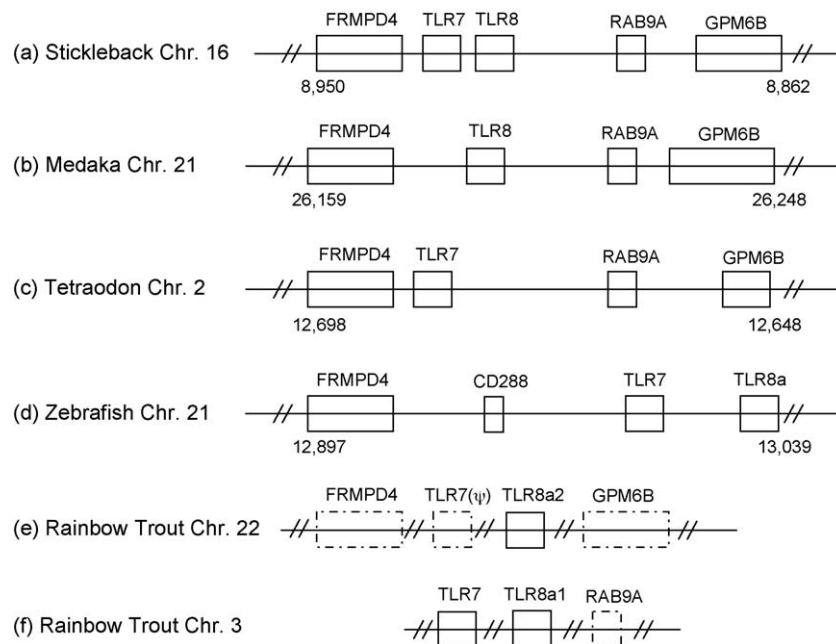


Fig. 2. Local genome synteny of chromosome fragments containing the *TLR7* and *TLR8* genomic region between five teleost species. Gene order and distances for stickleback, medaka, Tetraodon and zebrafish were from the Ensemble Genome Browser (<http://www.ensembl.org/index.html>; Release 55). The rainbow trout information is from partial sequencing of BAC clones 141K11 (*TLR8a2-TLR7ψ*) and 318O13 (*TLR8a1-TLR7*) from the Swanson 10× library and these BAC clones were mapped to chromosomes 22 and 3, respectively. The chromosome fragments are not drawn to scale. Genes predicted based on partial genomic sequence rather than complete open reading frame are marked by dashed frame. Physical distances are in kb, where available.

closely linked to OMM1728 ($r = 0.03$; LOD score of 41). Marker OMM3184 (Accession GF100700) was isolated from BAC 318O13 near *omTLR8a1* and was mapped to chromosome 3. It was closely linked to OMM1337 and OMM5005 ($r = 0.0$; LOD scores of 15 and 8). OMM5005 (Accession CO805111) [51] has high BLASTX sequence similarity to a Tetraodon ORF (Accession CAG05782) located on chromosome 2 between 11,025 and 11,028 kb of the Tetraodon genome sequence. The two trout *TLR8* loci were also linked to the *TNFSF13b* (*BAFF* [52]) (Accession DQ218467) homeologous regions on chromosomes 22 and 3 by way of their linkage to OMM3133 (Accession BV683032) on chromosome 22 and OMM3120 (Accession BV683031) on chromosome 3 [34].

BLASTX analysis revealed conserved synteny between the *TLR7/8* regions on the rainbow trout chromosomes 22 and 3 and the stickleback chromosome 16, medaka chromosome 21, Tetraodon chromosome 2 and zebrafish chromosome 21 (Fig. 2). Significant BLASTX similarities were found between two sequences from BAC 141K11 shotgun library (Accessions F1978330 and F1978331) and *FRMPD4* and *GPM6B* ORFs from the genomes of the four model teleosts. Similarly, significant BLASTX hits were found between Accession F1978371 from BAC 318O13 shotgun library and *RAB9A* ORFs from the genomes of those four teleosts.

3.2. Comparative domain organization of *omTLR7* and *omTLR8a1/2*

Alignments of *omTLR7* predicted peptide sequence with the human and *Fugu* *TLR7* (Fig. 3a) and *omTLR8a1* and *omTLR8a2* with the human, *Fugu* and Atlantic salmon *TLR8* (Fig. 3b) illustrated that the conserved extracellular CXRCXXXXPCXXC motif required for stimulus induced signal transduction and four highly conserved acidic residues critical for receptor function [13] were also present in rainbow trout. Likewise, three conserved TIR motifs important in signaling and receptor localization [2] were identified in the trout *TLR7* and *TLR8* amino acid sequences (Fig. 3). Computer analysis of *omTLR7* domains structure predicted 15 LRRs, including the N- and C-terminal regions and transmembrane and TIR domains. A signal

peptide was also predicted in the N-terminus of the receptor. The trout *TLR7* LRRs were distributed similarly to the LRRs identified in *Fugu*, zebrafish and human, although the precise positioning of some LRRs was distinct for each species (Fig. 4a). A similar picture was revealed from the analysis of *omTLR8a1* and *omTLR8a2* domains structure, but the variation in the number of LRRs was greater than *TLR7*. The number of LRRs varied from 14 in *omTLR8a2* to 16 in *omTLR8a1* and up to 18 in the *Fugu* and human *TLR8* peptides (Fig. 4b). Using the program SMART, a signal peptide was predicted for *omTLR8a1* but not for *omTLR8a2* and the Atlantic salmon *TLR8*. However, the program SignalP 3.0 predicted signal peptides for all three putative proteins with a probability of at least 95%.

3.3. Constitutive expression patterns of *omTLR7*, *omTLR8a1* and *omTLR8a2* mRNAs

All tissues tested had constitutive expression of *omTLR7*, *omTLR8a1* and *omTLR8a2*. The data were calibrated against expression level in the white muscle as the lowest expression was observed in this tissue. *TLR7* was highly expressed in the spleen followed by the anterior and posterior kidney tissues (Fig. 5a). *TLR8a2* was also highly expressed in the spleen and kidneys but also in a range of other tissues (Fig. 5b). *TLR8a1* had high expression in the spleen and kidneys and intermediate level in the gill, intestine, fat and skin (Fig. 5c).

3.4. Up-regulation of cytokine mRNAs in response to *TLR7* and *TLR8* agonists

In rainbow trout, poly I:C is known to up-regulate the type I interferon (IFN) genes while R848 up-regulates both type I IFN and pro-inflammatory cytokines genes [49,53]. In mammals, poly I:C binds to *TLR3* while R848 is an imidazoquinoline that signals through *TLR7/8* and is a potent inducer of anti-viral immunity [54,55]. Chloroquine, which inhibits endosomal acidification and maturation, can block activation of *TLR7* signaling by R848 in

human NK and 293-HEK cells and in chicken splenocytes [56–58]. We investigated whether pre-treatment of cells with chloroquine alters cytokine mRNA expression of *in vitro* stimulated leukocytes. Anterior kidney leukocyte cultures were stimulated with an optimal concentrations of poly I:C (5 µg/mL) or R848 (10 µg/mL) in the presence or absence of chloroquine (0.5 µM). *IFN1* and *IFN2* transcript levels did significantly differ among treatments in experiment 1 ($P=0.04$ and 0.0003 , respectively) but not in experiment 2; consequently, no post hoc tests between groups were conducted for experiment 2. A 6 h stimulation with poly I:C resulted in a non-significant up-regulation of *IFN1* expression (Fig. 6a). This up-regulation of *IFN1* by poly I:C was consistently but not significantly inhibited by chloroquine (69% inhibition). R848 also non-significantly up-regulated *IFN1* expression and the effect of chloroquine was not consistent (Fig. 6a). *IFN2* expression was significantly up-regulated by poly I:C in experiment 1 and there was a significant inhibition of this response by chloroquine (82% inhibition); the same trend was observed in experiment 2 but the

changes were non-significant (Fig. 6b). R848 moderately up-regulated *IFN2* and chloroquine did appear to have a non-significant inhibitory effect on this up-regulation (50% inhibition) (Fig. 6b). The two pro-inflammatory cytokines *IL-1β* and *IL-8* were significantly up-regulated by R848 (Fig. 6c and d). There was no evidence of chloroquine inhibition of *IL-1β* induction by R848, and the inhibition of *IL-8* expression by chloroquine was partial and not significant (42% and 15% inhibition in experiments 1 and 2, respectively). These data indicate that while R848 induces robust increases in cytokine mRNA abundance, the action is largely independent of the presence of 0.5 µM chloroquine.

The expression of the *TLR7* and *TLR8a1* genes were not significantly regulated by poly I:C or R848 stimulation (Fig. 7a and c). *TLR8a2* expression was down-regulated by R848 (with or without chloroquine) although this down-regulation was not significantly different from the media control (Fig. 7b). *TLR3* was significantly up-regulated 4.5-fold by poly I:C (with or without chloroquine) (data not shown).



Fig. 3. Amino acid sequence alignment of *omTLR7* with *Fugu* (fr) and human (hs) *TLR7* (A), and *omTLR8a1* with *omTLR8a2*, *Fugu*, Atlantic salmon (ss) and human *TLR8* (B). Alignment was performed using ClustalW v2.0 and edited manually. Alignment gaps are marked by dashes in the amino acid sequences. Asterisks (*) and colons (:) below the alignments indicate identity and similarity respectively for all species examined. The *omTLR7* (A) and *omTLR8a1* (B) leucine-rich repeats (LRR) are marked in grey shading and the transmembrane and Toll/IL-1 receptor (TIR) domains are underlined. The domains are labeled above the alignments. The extracellular CXRCXXXXPCXXC motifs important for the receptors function are boxed and in bold letters are the conserved acidic residues at the human *TLR7* positions 548, 565, 574 and 582 and *TLR8* positions 536, 543, 552 and 560. Highly conserved TIR amino acids important for signaling and receptor localization are highlighted in bold letters. NT: N (nitrogen/amino)-terminal. CT: C (carboxyl)-terminal.

(A)	
LRR-8	
omTlr7	SLNGSEFTFLTNLQYLDFFSNRLDLLYSSAFQELRSLVILDISNNHYFESEGLTHMLNF 589
frTLR7	SLNGSEFTYLTGLQYLDFFSNRLDLLYSTAFQELKKNLVILDISNNHYFESEGLTHMLNF 589
hsTLR7	TLNGSEFQPLAELRYLDFFSNRLDLLHSTAFELHKLKLEVLDISNSHYFQSEGITHMLNF 591
:***** *:***** *****:*****:*****:*****:*****:*****:*****	
LRR-9	
omTlr7	TKNLTLNLKILLMNYNKISTSTNTELESLSLEKLEFKGNRLDMLWRDGDTRYIDYFKKLLN 649
frTLR7	TQNLKKNLKLMMNHKISTSTNTELESQSLELEFRGNRLDMLWRDGNTRYLNFKKLSN 649
hsTLR7	TKNLKVLQKLMMNDNDISSSTRTMESESLRTELEFRGNHLDVLWREGDNRYLQLFKNLLK 651
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LRR-10 LRR-11 LRR-12	
omTlr7	LRVLDISHNNLNFIPQQVFQGLPDKLTNLYINNNRLKIFSWEKLIILQYLEVLDLSSNSI 709
frTLR7	LAVLDISHNNLNSIQQQVFSGLPDTLSELYVNNNKLKSFANGELSLPSSLVLDSLGNLY 709
hsTLR7	LEELDISKNSLSFLPSGVFDGMPNKLNSLAKNGLKSFWSKKLQCLKNLETLDLSHNQL 711
* *****:*****:*****:*****:*****:*****:*****:*****:*****	
LRR-12 LRR-13 LRR-14	
omTlr7	STVPPELSNCCTKSLKTLRLKKNQISKLSYFLKDAFSLKNLDLSFNNIQIEQTSIPDDV 769
frTLR7	TTVPHMLSNCSKSLKLLHKNELKLTDPFLKYAYNLKYLDLSFNRIQIEKSFPDDV 769
hsTLR7	TTVPERLSNCSRLKNLILKKNQIRSLTKYFLQDAFQLRYLDLSSNKIQMIQKTSFPENV 771
:*** *****:*****:*****:*****:*****:*****:*****:*****:*****	
LRR-CT	
omTlr7	VDQMDTLVLNNKFCMCNCNALFMVWMLNRTMVNIPLATAVTCAAPGAQRGHPVISLDLE 829
frTLR7	VNGMDMLLLHKNRFQSCSAIWFVWMLNQTTVTIPRLATDVTCASPGAQRGHPVISVD-- 827
hsTLR7	LNNLKMILLHNNRFLCTCDAVWFVWVNHTEVTIPYLATDVTCVGPAGHKQSISLD-- 829
:: .: *****:*****:*****:*****:*****:*****:*****:*****:*****	
transmembrane	
omTlr7	LQACQHNYSIILYILLTSLVLSFIASISSHLFLWDVWYLYHFLQAKFKGYRRLLSSPST 889
frTLR7	LLACQHNFLSIILYFLMTSFTLIFFTLAISSHLFLWDVWYIYHFCRAKLKGYSQLSSQSS 887
hsTLR7	LYTCELDLTNLILFSLSISVSLFVMVMTASHLYFWDVWYIYHFCRAKIKGYQRLISPCD 889
* **: : .:***: * *. * :.: : *****:*****:*****:*****:*****:*****	
TIR	
omTlr7	AYDAFVVYDKKDPEVSEWVLKELLVQLXNQGDHPLQLCLEERDWIPGCPIDNLSQSIHQ 949
frTLR7	VYDAFVMYDKEDSAVSEWVMKEMCVQLEGREDRRLTLCLEERDWIPGTPVIDNLSQSIHR 947
hsTLR7	CYDAFIVYDTKDPVTEWVLAELVAKLEDPREKHFNLCLEERDWLPGPVLENLSQSIQL 949
*****:*****:*****:*****:*****:*****:*****:*****:*****:*****	
TIR	
omTlr7	XXRTVFILTNTKYIKSGDFKTAIFYMAHQRLMDERDDVIVLIFLEKVPSSHXYLRRLRRLYR 1009
frTLR7	SKRTVFILTNTYIKSGSFKTAIFYMAHQRLMDKENDVIVLIFLENIPYNSRYLRRLRRLYR 1007
hsTLR7	SKKTVFVMTDKYAKTENFKIAFYLSHQRLMDKVDVILIFLEKPPQSKFLQLRRLCG 1009
:*****:*****:*****:*****:*****:*****:*****:*****:*****:*****	
TIR	
omTlr7	RSVMEWPTNPQAQQYFWFSLRSLVLTDSQKQYSNLFKETL 1049
frTLR7	RSVLEWPTNPQARPYFWFSVRSLVLTESHKQYNNLFKETL 1047
hsTLR7	SSVLEWPTNPQAHFYFWQCLKNALATDNHVAYSQVFKETV 1049
*:*****:*****:*****:*****:*****:*****:*****:*****:*****:*****	

Fig. 3. (Continued)

4. Discussion

In mammals, *TLR7* and *TLR8* are activated by synthetic anti-viral imidazoquinoline compounds and recognize single-stranded RNA (ssRNA) of viral origin, resulting in the induction of signaling pathways that activate production of various inflammatory cytokines [9,54,55,59]. In fish, *TLR7* and *TLR8* orthologs were identified in the zebrafish and *Fugu* genomes [7,17–19] and recently a *TLR8* ortholog was identified in Atlantic salmon [50]. In this study we identified two putative intact *TLR8* genes and a single intact *TLR7* gene in the rainbow trout genome and characterized their genomic location, gene structure, tissue expression distribution, and induced expression upon activation with synthetic molecular ligands.

The *TLR7/8* locus is highly conserved in vertebrates. *TLR7* and *TLR8* genes, or pseudogenes, are located in tandem on the same chromosome in mammals, birds and all fish sequenced to date [7,8,57] (Fig. 2; <http://www.ensembl.org/>). Therefore, it is likely that this tandem duplication event predated the split between the teleost lineage and the lobe finned lineage (which includes the lineage leading to tetrapods) some 400–450 million years ago (mya) [60,61]. The genomic organization of this region in rainbow trout exhibits conserved synteny with other fish genomes that extends beyond the *TLR7/8* tandem to the flanking genes (Fig. 2).

In rainbow trout the *TLR7/8* locus is duplicated as evident from BAC physical mapping and genetic linkage analyses. Analysis of DNA fingerprints of BACs that harbor the putative *omTLR8* genes resulted in two contigs, indicating that we have detected two gene loci [29,30]. Genetic linkage mapping of two microsatellite markers that we isolated from the two loci placed them on homeologous regions of chromosomes 3 and 22 which are flanked by the same duplicated genes and genetic markers [34]. Therefore, the *TLR7/8* locus duplication in rainbow trout was likely caused by the whole genome duplication event that occurred in the lineage leading to salmonid fishes some 50–100 mya [62]. We designated the intact putative *TLR8* genes from chromosome 22 *omTLR8a2* (Accession GQ422120) and from chromosome 3 *omTLR8a1* (Accession GQ422121). The Atlantic salmon *TLR8* [50] and *omTLR8a1* have 95% amino acid sequence similarity (Table 1) and were clustered together in our phylogenetic tree (Fig. 1). This suggests that they are true orthologs and that the ortholog of *omTLR8a2* has yet to be identified in Atlantic salmon. However, it is also possible that the ortholog of *omTLR8a2* was lost in Atlantic salmon through the evolutionary process of differential gene loss that frequently followed genome duplication events [63–65]. An example of differential gene gain/loss between rainbow trout and Atlantic salmon was recently identified in the MHC class IB region [66]. Rainbow trout IB region contains four functional non-classical

(B)	LRR-8	
omTlr8a1	FRKFS--HLKLIYLAENRLYPVSVSDSPGRVLGNSKSSELHKSPLTSHYSHSMDFSFELK	464
ssTLR8	FRKFS--HLKLIYLAENRLYPVSVSDSPGRVLGNSKNPALHKSPLTSHYSHSKDFFSFLK	463
omTLR8a2	FNKSNSSLLKYIYLAENRLYPISVNESPGCGTGNNLSKVLYTSLPTGYYSNRDPSYGIN	462
frTLR8	FKKLS--HLKMIYLGENRLYPEKNMPS--LSRQYNQLDFSVSSYMKPHGRDYFFKIS	452
hsTLR8	FQNFS--NLEIIYLSSENRI SPLVDTRQSYANSSSFQRHIRKRRTDFEFDPHSNFYHT	471
	.:: *.:*: *.:*: *.:*: *.:*: *.:*: *.:*: *.:*: *.:*: *.:*: *	
	LRR-9	
omTlr8a1	HGLVKQEFCFDSGRVLVLSSNNIFFISPQKFEGYGDIACLNLSRNGFSAALNGTEFTTLPA	524
ssTLR8	HGLVKQEFCFDSGRVLVLSSNNIFFISPQKFEGYGDIACLNLSRNGFSAALNGTEFTTLPA	523
omTLR8a2	HNLVKPECFISGRVLVLSSNNLFFISPQKFEGYGDIACLNLSRNGFSAALNGTEFTSLPN	522
frTLR8	QTLIKQECPDAGKVLSSLNNSNLFISTKEFEG--DIKCLNLSRNGFAPALNGTEFTSLPN	510
hsTLR8	RPLIKPQCAAYGKALDSLNSIFFIGPNQFENLPDIACLNLSANSNAQVLSTGEFSAIPH	53
	: *	
	LRR-10	LRR-11
omTlr8a1	LTYYLDLSFNKIDLAYNNAFKELHKLQVLDSLNSHYFEVSGVTNHNFLTNPALKVLNM	584
ssTLR8	LTYYLDLSFNKIDLAYNNAFKELHKLQVLDSLNSHYFEVSGVTNHNFLTNPALKVLNM	583
omTLR8a2	LKYYLDLSFNKIDLAYDNFAKELKKLEILDLSYNPHYFEVSGVTNHNFLTNPALKVLNM	582
frTLR8	LTYYLDLSFNKIDLAYSLAFNDLKKLQVLDSLNSHYFNVQGITHKVNFQRLNLPVLVLM	570
hsTLR8	VKYYLDLTNNRLDFNASALTDEVLDSLNSHYFRIAQGVIHHLEFIQNTLNKLVNL	591
	: .:*. : *	
	LRR-11	LRR-12
omTlr8a1	SNNKIPTLTTKQ-MTSASLKELQPRHNXLATLWKERDASYNRLFKNLVNLTYLDISFNSI	643
ssTLR8	SNNNIPTLTTKQ-MTSASLKELQPHNLLATLWKERDGSYNLFLKNLVNLTYLDISFNSI	642
omTLR8a2	SHNNIPTLTTKQ-MTSTSLKELQPHNLLATLWKEGDDSYNLFKKTNLTYLDISFNSI	641
frTLR8	SHNDISTLTTKY-MESKSLAELRPTHNYLGTLWKENDLSYKKLFTKLTNLTYLDISFNQI	629
hsTLR8	SHNNIYTLTDKYNLESKSLVELFSGNRLDLWNDDNRYISIFPKKNLTRLDSLNLRL	651
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	LRR-12	LRR-13
omTlr8a1	EKIPSTVYDNLPTYTLQKLCISHNLLTHFDWDKLASFQQNLNLDLSYNLYHMSANLSNFT	703
ssTLR8	EKIPSTVYENLPNTLQKLCISHNLLTHFDWDKLASFQQNLNLDLSYNLYHMSANLSNFT	702
omTLR8a2	EKIPSKVYENIPYTTLQKLCISHNSLCHFDWDKLAFQQNLFLDXSYNSLFHVASANLSNFT	701
frTLR8	EKIPDEMAYKHPQNLTTLLISHNFLDPFKWNKILFLPQIKVLDLSFNRLTNVTG--IHIA	687
hsTLR8	KHIPNEAFNLNLPASLTTELHINDNMLPFNWTLQQFPRLLEDLRGNKLLFLTPDLSDF	711
	: *. : *: *: *: *. : *: *. : *: *. : *: *. : *: *. : *: *: *: *: *	
	LRR-14	LRR-15
omTlr8a1	NALQMLDLSYNQIAQLSDGLFGKAH-LQLLDLSHNQLTIVNETTFLGPENYMKTLSQLG	762
ssTLR8	NALQMLDLSYNQIAQLSDGLFGKAQSLQILDLSHNQLTIIINETTFLGSPENYMKTLSQLG	762
omTLR8a2	DTLQILDLSHNQIFQLSRGLFGAQSLQILDLSYNQLTIIINETTFLGSPENYMKTLSQLG	761
frTLR8	HTLLTLNLKHNGIGHLLDGGFLMGAKRLQVLNLSNQLTINESTQPRENQFTLYLEE	747
hsTLR8	SSLRTLSSHNRISHLPSPGLSEVSLLKHLDDLSSNNLKTINKSALETKTITTKLSMLELHG	771
	: * *: *. : *: *. : *: *. : *: *. : *: *. : *: *. : *: *. : *: *	
	LRR_CT	
omTlr8a1	NPFQCTCDLDFDIMWIKDNKVEIPGLATELICNMMPAETRGQPVILFDIGECVNDVNAFL	822
ssTLR8	NPFQCTCDLLEFLMWIKDNKDIPIRLATELICNMMPAKMKGPVILFDKKECLNVNKAFQ	821
omTLR8a2	NPFQCTCDLQEFIQWIKDNKNVEIPRLASEVTCNMMPAKMRGQPMILFINKECNDNIAFL	822
frTLR8	NPFQCTCDLLDFILWIE--NSDVKIPGLATDVCKADAPANQKGVRVLKFDITCVNNSEAF	806
hsTLR8	NPFECTCDIGDFRRWMDEHLNVKIPRLV-DVICASPGDQRGKSIVSLELTTCVSDVTAVI	830
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	Transmembrane	TIR
omTlr8a1	IYLSLASLIMLTMVITMAAHMFYWDASYILYILRAKLKGYHSFGSATNNLYGAFVITYDT	882
ssTLR8	IYLSLSTSLIMLTMVITMAAHMFYWDASYMYLYLRVLKLGYSFHSRATTNLYDAFVITYDT	882
omTLR8a2	IYLSLSTSLIMFTVMVITMAGHFYWDASYILYILRAKLKGYHSLKSTTTNLYDAFVITYDT	881
frTLR8	IYILTSSSFIGFMVLTVTAHLVFWDATVLYHYMKAKLKGYSLLNSS--EILYGAFTVITYDT	864
hsTLR8	LFFFTTFITTMVMLAALAHHLFVYDWVP IYNVCLAKVGYSRLSTS--QTIFYDAYISYDT	888
	: *	
	TIR	
omTlr8a1	RDLWVSDVWLNLHRVQLEERQERHPLCLEERDWPVGPLIDNLNLSIRQSRKTVFVLTE	942
ssTLR8	RDLLVSDVWLNLHRVQLEERQERHPLCLEERDWIPGVPLIDNLSHSIRQSRKTVFVLTE	942
omTLR8a2	RDPLVSDVSWLNQLRVQLEERGHRHPLCLEERDWAPGVPLIDNLFQSIRQSRKTVFVLTE	941
frTLR8	RDPHSVSEWMKNLLVLEEAGEKNLPLCLEERDWTGPVPLVDNLTSIRYSRKTVFLVLTQ	924
hsTLR8	KDASVTDVWINELRYHLEESRDKNVLLCLEERDWDPLGLAIIDNMQSINQSKKTVFVLTK	948
	: * *:*. : *	
	TIR	
omTlr8a1	AIVRTGTFRMAVYLAHQRLDENMDVIVLVLEPVLQHSFHLRLRRRLCGSVLEWTRTA	1002
ssTLR8	AIVRTGTFRMAVYLAHQRLDENMDVIVLVLEPVLQHSFHLRLRRRLCGSVLEWTRTA	1002
omTLR8a2	AIYRTGNFRMAVYLAHQRLDENIDVIVLVLEPVLQHSFHLRLRRRLCGRSVLEWPQA	1001
frTLR8	DYVKGTGIKAMVYLAHQRLDENVDVIVLLLEPVLQHSFHLRLRRRLCGSVLEWPRTA	984
hsTLR8	KYAKSWNFKTAFLALQRLMDENMDVIIIFILLEPVLQHSQYLRRLQRICKSSILQWPDNP	1008
	* :*: *: *. : *: *. : *: *. : *: *. : *: *. : *: *. : *: *. : *: *	
	TIR	
omTlr8a1	AAEPFWFQCLRNAVRLDNQVMYNQMYSRYFTNK 1035	
ssTLR8	AAEPFWFQCLRNAVSLDNQVMYNQMYSRYFTNK 1035	
omTLR8a2	AAETWFQWHLRNARVRLDNQVMYNKYSISRYFTNK 1034	
frTLR8	AAEPFWFQNLARNVVRVENQVMYNKNYSKYFTSK 1017	
hsTLR8	KAEGFLFWQTLARNVVLTDNSRYNNMVVDSISKQY 1041	
	** ***** * :* * :* * :* * :* * :* * :* * :* * :* * :* *	

Fig. 3. (Continued).

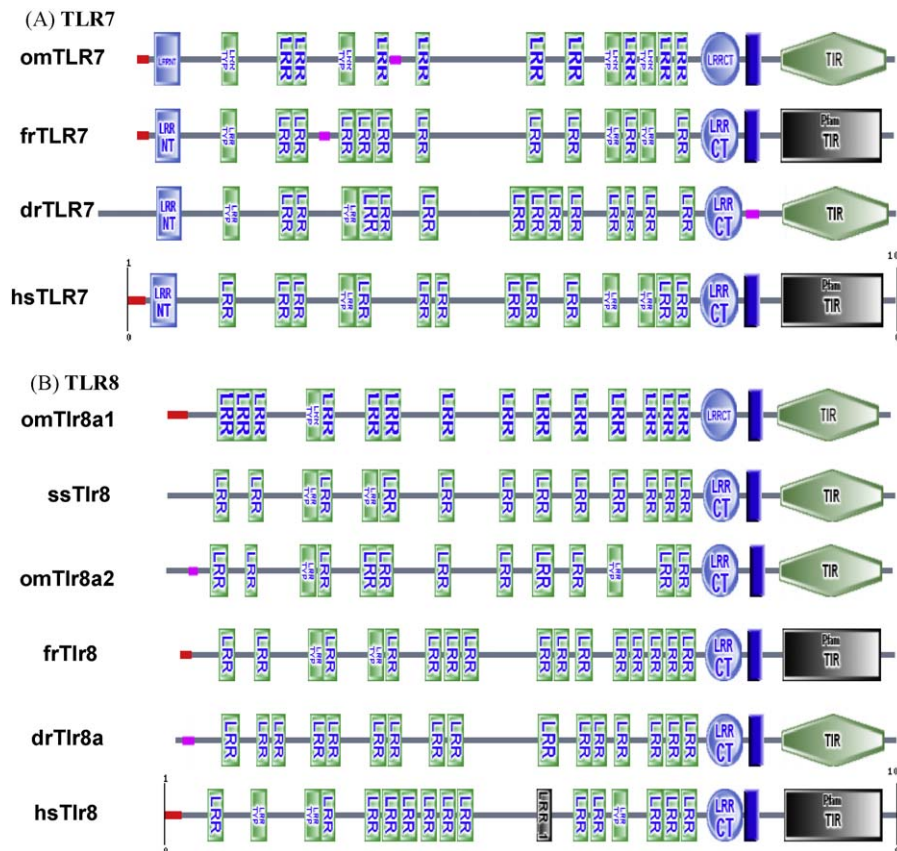


Fig. 4. Schematic comparative domain organization of *omTLR7* with *Fugu* (fr), zebrafish (dr) and human (hs) *TLR7* (A), and of *omTLR8a1* and *omTLR8a2* with zebrafish (dr) *TLR8a* and Atlantic salmon (ss), *Fugu* (fr), and human (hs) *TLR8* (B). The domain organization of each TLR was predicted using the SMART program analysis tool (<http://smart.embl-heidelberg.de/>). LRR: leucine-rich repeat; LRR_TYP: leucine-rich repeat typical; TIR: Toll/IL-1 receptor; NT: N (nitrogen/amino)-terminal; CT: C (carboxyl)-terminal; (■) transmembrane domain; (■) low compositional complexity segment; (■) signal peptide.

Alignment of the rainbow trout and human *TLR7* and *TLR8* amino acid sequences revealed high conservation of motifs that were identified as critical for the receptors function in human. The TIR domain harbors amino acids known to play important role both in the receptor signaling and intracellular localization. Three conserved TIR motifs important in signaling and receptor localization in human [2] were also identified in the trout *TLR7* and *TLR8* amino acid sequences (Fig. 3). Conserved features in the ectodomain of human *TLR7* and *TLR8* that are essential for pH-dependent signal transduction [13] were also found in the rainbow trout predicted sequences. The CXRCXXXXPCXXC conserved sequence motif was detected between amino acid positions 251 and 264 of *omTLR7*, 251–264 of *omTLR8a1* and 247–260 of *omTLR8a2*. The human *TLR8* highly conserved acidic side chains Asp⁵³⁶, Asp⁵⁴³, Glu⁵⁵² and Asp⁵⁶⁰ were also found in *omTLR7* (Asp⁵⁴⁶, Asp⁵⁶³, Glu⁵⁷² and Asp⁵⁸⁰), *omTLR8a1* (Asp⁵²⁹, Asp⁵³⁶, Glu⁵⁴⁵ and Asp⁵⁵³) and *8a2* (Asp⁵²⁷, Asp⁵³⁴, Glu⁵⁴³ and Asp⁵⁵¹) (Fig. 3). Further analysis is needed to explore the physiological significance of the lack of conservation of human *TLR7* Glu⁵⁸⁰ and partial conservation of human *TLR8* Glu⁵⁵⁷ in teleosts.

The tissue expression profiles of *omTLR7*, *omTLR8a1* and *omTLR8a2* were similar as all three showed strongest expression in the spleen followed by still relatively high expression in the anterior and then posterior kidney (Fig. 5). Measurable expression was observed in all tissues tested for all three genes, but the relatively high expression in the spleen was more pronounced in *omTLR7* (~250-fold relative to white muscle) than in *omTLR8a2* (~75-fold) and *omTLR8a1* (~65-fold). Direct comparison of the rainbow trout expression profiles to similar studies in zebrafish

[17], *Fugu* [19] and Atlantic salmon [50] is confounded by our use of the more sensitive quantitative PCR, which likely contributed to the detection of a base-line level in all tissues in rainbow trout. In *Fugu* the *TLR7* expression was restricted to kidney, heart and gill with stronger expression in the kidney and no expression in the spleen, and *TLR8* was expressed in a wide range of tissues. In zebrafish, both genes were expressed in a wide range of tissues, but while *TLR7* was expressed in the spleen, *TLR8* was not. In Atlantic salmon *TLR8* expression was restricted to the spleen, anterior kidney and gills, with highest expression in the spleen. Similar differences between species expression profiles were previously observed for *TLR3* in fish and in mammals [24]. Comparison of *TLR* genes expression should be interpreted with caution since such discrepancies may result not only from species variation but also from differences in immunological status, developmental stage [74], and genetic background.

Diverse *TLR7/8* agonists' specificity and differences in species cytokine transcription response repertoire were previously reported. In human, *TLR7* and *TLR8* have differential agonist specificity and in mice *TLR8* does not respond to the human *TLR8*-specific ssRNA agonists [58,59]. In chicken, a broad range of human *TLR7* agonists failed to induce up-regulation of *IFN-α* or *IFN-β*, but induced up-regulation of pro-inflammatory cytokines like *IL-1β* and *IL-8* [57]. In rainbow trout, poly I:C was previously shown to up-regulate type I *IFN* genes and R848 up-regulated both type I *IFN* and pro-inflammatory cytokines [49,53]. Stimulation of *IFN*-like cytokines and *IL-1β* by the *TLR9* agonist CpG oligodeoxynucleotide in rainbow trout anterior kidney leukocytes was previously shown to be suppressed by chloroquine, a compound known to block

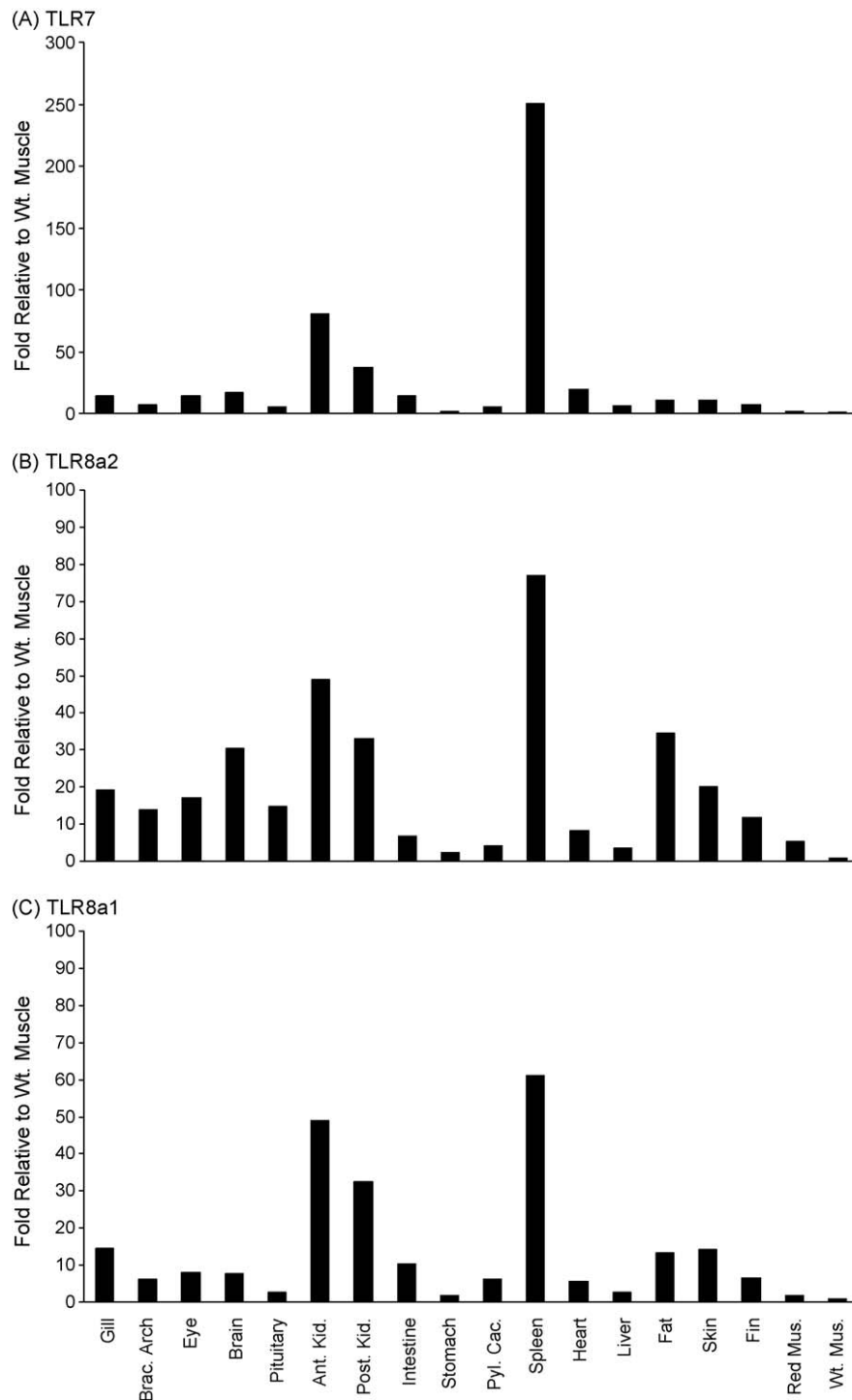


Fig. 5. Gene expression of TLRs in a range of tissues sampled from naive rainbow trout as measured by quantitative RT-PCR. Figures represent expression of pooled RNA samples from five rainbow trout. Genes are as follows: (A) *TLR7*, (B) *TLR8a2* and (C) *TLR8a1*. All data were normalized to the housekeeping gene *EF1- α* and data are presented as fold-change relative to white muscle (the tissue with the lowest expression). The white muscle CT values of *TLR7*, *8a2* and *8a1* expression were 36.0, 32.2 and 31.9, respectively. Tissue abbreviations are: brac. arch (brachial arch), ant. kid. (anterior kidney), post. kid. (posterior kidney), pyl. cac. (pyloric caca), red mus. (red muscle) and wt. mus. (white muscle).

endosomal acidification and inhibit endosomal maturation [46]. In this study, rainbow trout anterior kidney leukocytes were stimulated with the *TLR3* agonist poly I:C or the *TLR7/8* agonist R848 in the presence or absence of chloroquine. Transcription of the type I IFN genes *IFN1* and 2 was up-regulated by both poly I:C and R848, but only *IFN2* expression induced by poly I:C was suppressed in the presence of chloroquine (Fig. 6b). This implicated

endosomal localization of *TLR3* in trout leukocytes is consistent with similar findings in *Fugu* [16]. The two pro-inflammatory cytokines were up-regulated by R848, but the effect of chloroquine was not consistent. *IL-1 β* was not affected by chloroquine while *IL-8* inhibition by chloroquine was not significant (42% and 15% inhibition in experiments 1 and 2, respectively) (Fig. 6d). Our results suggest that the rainbow trout cytokine transcription

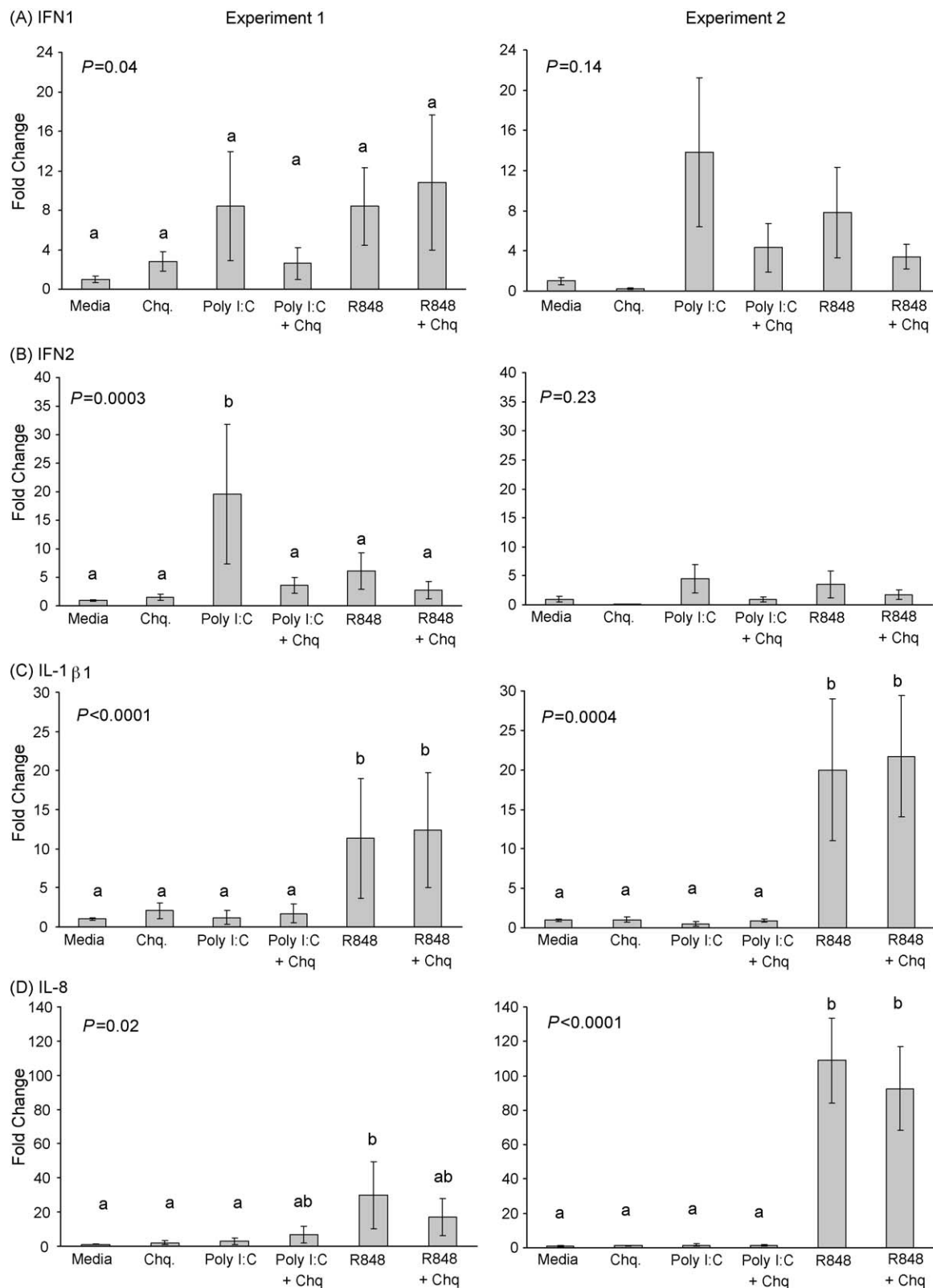


Fig. 6. Cytokine expression in stimulated anterior kidney leukocytes as measured by quantitative RT-PCR. Two independent leukocyte experiments were conducted (experiments 1 and 2). Cells were stimulated with media, chloroquine alone, poly I:C alone, poly I:C in the presence of chloroquine, R848 alone or R848 in the presence of chloroquine. All data were normalized to the housekeeping gene *ARP* and data are presented as mean fold-change relative to the media control \pm 1 standard error (mean of 4 fish per experiment). Genes are as follows: (A) *interferon (IFN) 1*, (B) *IFN2*, (C) *Interleukin (IL) 1 beta-1* and *IL-8*. ANOVA was used to test for differences among all treatment groups for each gene; overall P -value is shown in the figure panel. Significant differences between groups detected by post hoc testing are designated with distinct letters shown in lower case. No post hoc testing was conducted if the ANOVA analysis was not significant.

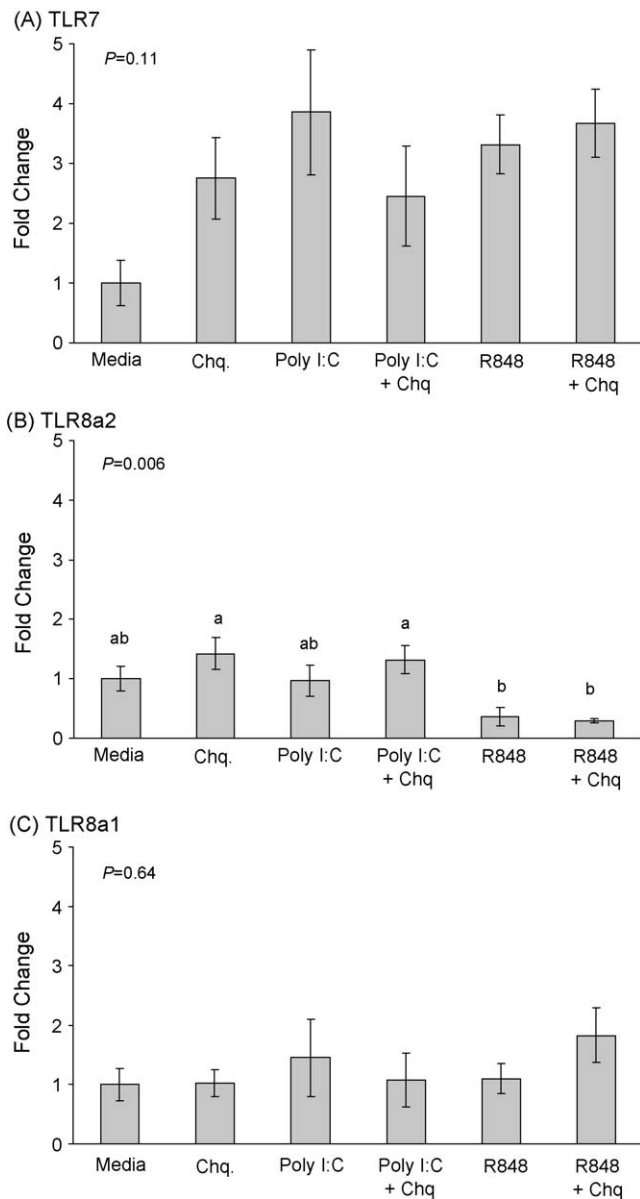


Fig. 7. TLR gene expression in stimulated anterior kidney leukocytes as measured by quantitative RT-PCR. Genes are as follows: (A) *TLR7*, (B) *TLR8a2* and (C) *TLR8a1*. Cells were stimulated with media, chloroquine alone, poly I:C alone, poly I:C in the presence of chloroquine, R848 alone or R848 in the presence of chloroquine. All data were normalized to the housekeeping gene *EF1-α* and data are presented as fold-change relative to the media control \pm standard error. ANOVA *P*-values are presented for each gene. Significant differences between groups detected by post hoc testing are designated with distinct letters shown in lower case. No post hoc testing was conducted if the ANOVA analysis was not significant.

response repertoire to R848 is likely more similar to the human response than to chicken, as type I IFN cytokines were induced by R848 in addition to pro-inflammatory cytokines. In addition, the rainbow trout *IL-1β* expression stimulated by R848 was not suppressed by chloroquine (Fig. 6c), where in chicken *IL-1β* expression from splenocytes stimulated by R848 was significantly suppressed in the presence of chloroquine [57]. These data suggest that either the dose of chloroquine we used (0.5 μ M) was not sufficient to inhibit R848 mediated signaling in trout cells or that fundamental differences in receptor localization or activation exist. Further work is necessary to identify *TLR7/8* localization within rainbow trout cells and their signal activation requirements.

In addition to up-regulation of secreted immune chemokines, agonists may also induce changes in the expression of the *TLR* genes [24,58]. *TLR7* and *TLR8a1* expression levels in rainbow trout anterior kidney leukocytes were not affected by poly I:C or R848 treatments, but surprisingly, *TLR8a2* expression was down-regulated by R848 (Fig. 7b). Previously we observed five-fold increase in *TLR3* expression in rainbow trout anterior kidney leukocytes stimulated with poly I:C for 6 h [24]. In this study we repeated the *TLR3* expression assay and observed similar results (4.5-fold increase; data not shown). Pre-treatment with chloroquine had no effect on the *TLR* genes expression. It is possible that longer incubation with R848 will result in significant up-regulation of *TLR7* or *TLR8a1* and even stronger down-regulation of *TLR8a2* as 12 h incubation with poly I:C previously resulted in 30-fold *TLR3* expression increase [24]. In Atlantic salmon, the ortholog of *omTLR8a1* was not up-regulated in the spleen of fish injected with an ssRNA virus although type I IFN and *Mx* genes were up-regulated [50]. The down-regulation of *omTLR8a2* may imply that this gene has evolved to a new or altered function as often occurs when two duplicated genes remain active [29,65,75,76]. Alternatively, this down-regulation may be the result of a negative feedback loop [77] and not an acquired new function.

Several lines of evidence were presented in this paper that the rainbow trout genes we identified are orthologs of the *TLR7* and *TLR8* genes. The genomic location of the duplicated trout *TLR7/8* locus exhibited conserved synteny with the genomes of other teleosts; the amino acid sequences and the predicted structural domains are highly conserved; the pattern of rainbow trout cytokines expression in response to the *TLR7/8* agonist R848 is similar to the human response. Further research is needed to better understand the ligand specificity and the roles of *TLR7* and *TLR8a1/2* in the rainbow trout immune response. The development of assays to measure ligand binding and downstream signaling is critical for future elucidation of *omTLR7* and *omTLR8a1/2* functions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dci.2009.10.002.

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